STUDY OF THE EXPRESSION PROFILE OF THE UGT1A1 GENE IN NEONATAL JAUNDICE PATIENTS

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CERTIFICATE

This is to be certify that **Mr. Somya Ranjan Patra** a second year student of Department of Life Science, NIT, Rourkela having Roll number 410LS2066 has carried out a dissertation project work titled "**Study of the expression profile of the UGT1A1 gene in neonatal jaundice patients**" under the guidance of **Dr. S. K. Patra,** Associate Professor and Head, Department of Life Science, National Institute of Technology (NIT), Rourkela.

He has taken greater accountability and in depth care for success of this project. The project is entirely satisfactory to the best of my knowledge.

Place:

Date: **Dr. S. K. Patra**

DECLARATION

I hereby declare that the dissertation project report entitled "**Study of the expression profile of the UGT1A1 gene in neonatal jaundice patients**" the is an authentic and original work done and submitted by me in partial fulfilment of the Degree of Master of Science in Life Science, NIT Rourkela and that this report, up to a substantial extent, has not been submitted or published elsewhere before, to the best of my knowledge. This is a report of work done by me under the guidance of **Dr. S. K. Patra** Associate Professor and Head, Department of Life Science, National Institute of Technology (NIT), Rourkela.

SOMYA RANJAN PATRA

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Abbreviations

UGT	uridinyl glucuronyl transferase.
STB	serum total bilirubin.
µmol/l	micromoral per litre
Mg/dL	milligram per decilitre
G-6-P D	glucose 6 phosphate dehydrogenase
HOXG	heme oxygenase
CO	carbon monoxide
Fe3+	ferric ion
kDa	kilo Dalton
µl	microliter
RBC	Red blood cells
cDNA	complementary DNA
rpm	revolutions per minute
KCl	potassium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
gms.	Grams

ABSTRACT

Diseases are either caused due to a genetic defect or physiological defects, however certain pathological disorders such as neonatal jaundice is a product of intereliant working of both these causal factors. The disease is characterized by hyperbillirubinemia and subsequent yellowing of the skin of the patient. The etiological causes are more related to the genetic and epigenetic factors i.e. the genes that are responsible in controlling the enzymes that play an important role in carrying out the processes of bilirubin excretion and the epigenetic modifications that regulate the transcriptional activity of those genes. The expression of the gene gets affected by either due to a mutation in the gene or due to a epigenetic change. One of the prominent enzymes responsible is UGT1A1 and lowering of its activity is a major factor contributing to this disease. Keeping this view in mind, the present study was carried out to study the expression profile of the UGT1A1 gene in neonatal jaundice patients and correlate it with epigenetic studies.

The project aims to find a definitive epigenetic cause directing the UGT1A1 dysregulation in neonatal jaundice so as to fully understand the disease. Further studies in this aspect will present avenues for drug development based on the mechanistic details of this enzyme.

Key words: hyperbillirubinemia, epigenetic factors, neonatal jaundice and UGT1A1 gene.

1. INTRODUCTION

Most of the diseases are either caused due to a genetic defect or a physiological defect but Neonatal jaundice is a disease that is caused due to either of the defects and both the aspects are inter-dependant i.e. dependent on each other . The former being rare but often fatal and the latter being more common and is rather a measure to acclimatize with the sudden change in the environment after birth. The disease is symptomized by hyperbillirubinemia and yellowing of the skin of the patient (baby) due to this. The pathological causes are more related to the genetic and epigenetic factors i.e. the genes that are responsible in controlling the enzymes that play an important role in carrying out the processes of bilirubin excretion as it is an unwanted poisonous substance for the body. This aspect is a very important to deal with as far as research in the field is concerned.

1.1. NEONATAL JAUNDICE

Neonatal jaundice or **Neonatal Hyperbillirubinemia** is yellowing of the skin and other tissues of a new born infant due to bilirubin deposition. A bilirubin level of more than 85 umol/l (5 mg/dL) manifests clinical jaundice in neonates whereas in adults a level of 34 umol/l (2 mg/dL) would make the skin look yellowish. In neonates jaundice is detected by blanching the skin with digital pressure so that it reveals underlying skin and subcutaneous tissue (Gartner and Herschel, 2001) and enables jaundice detection. Jaundice neonates have an apparent yellowish sclera, and face, extending down towards the chest. In neonates the dermal icterus is first noted in the face and as the bilirubin level rises it proceeds caudal to the trunk and then to the extremities which makes it appear on nail tips. This condition is common in neonates affecting over half (50 -60%) of all babies in the first week after birth. This condition is also called **Gilbert's syndrome**.

1.1.1. Types of Neonatal Jaundice.

There are two types of Neonatal jaundice basing upon the cause behind this condition.

1. Physiological: when hyperbillirubinemia or gilbert's syndrome happens due to any reason other than those concerning genetics or heredity and is caused due to a minor temporary defect during bilirubin excretion or conjugation is called Physiological Jaundice.

2. Pathological: when the disease is caused due any defect arising due to any deficiency of any vital enzyme due to a genetic defect that may not be reversible is called pathological jaundice.

1.1.2. Differentiating Physiological and Pathological Jaundice

The signs which help to differentiate pathological jaundice of neonates from physiological jaundice of neonates are presence of intrauterine retardation, stigma of intrauterine infections (e.g. cataracts, hepatosplenomegaly, microcephaly etc.), bruising, cephalhematoma, signs of intra ventricular haemorrhage etc. History of illness is to be taken note of. Family history of anaemia and jaundice, family history of neonatal or early infant death due to diseases of the liver, maternal illness are suggestive of viral infection (rash, fever, or lymphadenopathy), maternal drugs (like for e.g., anti-malarials, Sulphonamides causing haemolysis in G-6-PD deficiency) are suggestive of pathological jaundice in neonates (Barbara and Zhang *et al,* 2006).

1.2. CAUSES

In neonates, jaundice tends to develop because of two factors - the breakdown of fetal haemoglobin as it is replaced with adult haemoglobin and the relatively immature hepatic metabolic pathways which are unable to make bilirubin conjugated with glucuronic acid for transport so excrete bilirubin very slowly unlike adults. This causes an accumulation of bilirubin in the blood causing hyperbillirubinemia which leads to the symptoms of jaundice. This condition is also called Gilbert's syndrome.

Gilbert's syndrome is more of a phenotypic effect than a disease, characterized by mild jaundice due to an increased unconjugated bilirubin in the blood that makes it incompetent for transporting and ultimately gets excreted. This arises from several different genotypic variations of the gene controlling the enzyme responsible for changing bilirubin to the conjugated form (Agarwal and Chowdhury *et al,* 2005).

It is characterized by a 70%-80% reduction, rather than more severity in the loss of activity, in the glucuronidation function of an enzyme known as uridine-diphosphate-glucuronosyltransferase isoform 1A1 (UDP-glucuronosyl transferase 1A1 or UGT1A1) (Chang *et al*, 2003). The **UGT1A1 gene** is located on the chromosome 2 in humans. There are considerably more than 100 variants of the UGT1A1 gene found in a mammalian chromosome, designated as UGT1A1*n ('n' is the general chronological order of discovery), either of the gene itself or of the promoter region of the gene. The UGT1A1 gene is always associated with a TATA box promoter region. This region normally contains the genetic sequence $A(TA_6)TAA$; this variant accounts for more than 50% of alleles in many populations in North Africa and Asia. However there are many allelic polymorphic variants of this region, the most common being adding another dinucleotide repeat TA to the promoter region, so that it is referred to as $A(TA_7)TAA$, and also being called as UGT1A1*28; this common variant accounts for more than 40% of alleles in some populations, but often it is seen less, approximately 3% of alleles, in southeast and east Asian populations and in the Pacific Islanders.

In most populations, Gilbert's syndrome is commonly associated with homozygous A (TA_7) TAA alleles. In 94% of GS cases, two other glucuronyltransferase enzymes, UGT1A6 (rendered 50% inactive) and UGT1A7 (rendered 83% ineffective), are also found to be affected.

However, Gilbert's syndrome can arise without TATA box promoter polymorphic mutations; in some populations, particularly in the southeast and east Asians and in parts of eastern Europe. Gilbert's syndrome is more often a consequence of heterozygote missense mutations (such as Gly71Arg also known as UGT1A1*6, Tyr486Asp or UGT1A1*7, Pro364Leu or UGT1A1*73) in the coding region of the actual gene, which may be associated with significantly higher bilirubin levels (Patra et al, 1997). Because of its effects on drugs, bilirubin breakdown and its genetic inheritance, Gilbert's syndrome can be classified into a minor inborn errors of metabolism. Basing upon the types bilirubin conjugation the causes can be divided into two types.

1.3. BILIRUBIN CONJUGATION

It is a phenomenon by which the bilirubin is conjugated with glucuronic acid in the liver by the enzyme glucuronyltransferase, making it soluble in the water constituent of the blood. Much of it goes into the bile and thus out into the small intestine. However 95% of the secreted bile is reabsorbed by the small intestine. This bile is then resecreted back into the small intestine by the liver. This process is called as enterohepatic circulation(Monaghan and McLellan, 1999). About half of the conjugated bilirubin remaining in the large intestine (about 5% of the original secretion) is metabolised by the colonic bacteria to urobilinogen, which can be further metabolized into stercobilinogen, and finally can be oxidised to **stercobilin**. Stercobilin gives faeces its brown colour. However, just like bile, some of the urobilinogen is reabsorbed, and 95% of the total reabsorbed is resecreted in to the bile which is also a part of enterohepatic circulation.

A small amount of the reabsorbed urobilinogen (about 5%) is excreted through urine in the form of urobilin formed due to oxidation, which gives urine its characteristic yellow colour. The whole process results in only less than 20% of the secreted bile being excreted in the faeces. The amount lost depends on the secretion rate of bile (Huang and Chang *et al*, 2002). Although the terms direct and indirect bilirubin are used equivalently with the terms conjugated and unconjugated bilirubin, they cannot be used in this manner as far as quantitative correctness is considered, it is because of the fact that the direct fraction includes both conjugated bilirubin and δ bilirubin (i.e. the bilirubin bound covalently to albumin, that appears in serum when hepatic excretion of conjugated bilirubin is not done properly in patients with hepatobiliary disease).

Furthermore, the direct bilirubin tends to overestimate the levels of conjugated bilirubin mostly due to unconjugated bilirubin that has already reacted with diazosulfanilic acid, leading to increased azobilirubin levels (and the increased direct bilirubin).

Objective.

To isolate m-RNA from the blood sample of neonatal jaundice patients and then study the expression profile of the UGT1A1 gene through cDNA synthesis and amplification.

2. REVIEW OF LITREATURE

2.1. NEONATAL JAUNDICE

Jaundice (predominantly an elevation of unconjugated bilirubin) occurs in about half of neonates during the first 5 days of life and is termed physiologic jaundice. Neonates often have delayed maturation of bilirubin uridine diphosphate–glucuronosyl transferase (UGT1A1) expression with normal activity attained by 3 months of age. In healthy term neonates serum total bilirubin levels rise to ~100 mmol/L within the first3 days (the normal adult STB level is 17 mmol/L) and it decreases within 7 to 10 days of accumulation. STB levels in breast-fed infants are higher, reaching upto 256 to 410 mmol/L within the first 10 to 20 days of life and breast milk jaundice occurs in 0.5% - 34% of breast-fed neonates. Pathological jaundice is diagnostically investigated generally in term neonates with STB levels >200 mmol/L. This is condition is also called type 1 Crigler-Najjar syndrome.

2.1.1. Breast-feeding and jaundice

Exclusively breast-fed infants when compared to artificially fed babies have a different pattern of physiological jaundice. Jaundice i.e. hyperbillirubinemia in breast-fed babies usually appears between 1-3 days of life, peaks during 5-15 days of life and then disappears by the third week of life. The breast-fed neonates have also been reported to have even higher bilirubin levels. Studies have revealed that one third of all breast-fed babies are detected to have mild clinical jaundice during the second and third week of their life, which may persist into the 2nd to 3rd month of life in a few neonates. Authors have stated that this increased frequency is related to the pattern of breast-feeding and not to the characteristics of breast milk. The decrease in frequency of breast-feeding is associated with exaggeration of physiological jaundice in neonates. Encouraging a mother to breastfeed her baby at least 10-15 times a day would be helpful in the management of jaundice in a term healthy baby (Hang and Chang *et al*, 2002).

2.1.2. Breast milk jaundice

Approximately 2-4% of exclusively breast-fed term babies have jaundice with more than 10 mg/dl of STB levels in the third week of life. These babies with STB beyond 10 mg/dl in the third week of life should be investigated for prolonged jaundice as this should not be the case in a normal baby. A diagnosis for breast milk jaundice should be done if the serum bilirubin is predominantly unconjugated(Gourley *et al*, 2002). Mothers should be advised to continue breast-feeding at more frequent intervals and bilirubin levels usually subside over a period of time. Interruption of breast-feeding should not be avoded unless levels exceed 20 mg/dl.

2.2. HEME DEGRADATION

Heme Degradation begins inside macrophages of the spleen, which plays a major role in removing old and damaged erythrocytes from the circulation. The heme degradation occurs in a step wise manner like other metabolic processes. In the first step, the heme disintegrated from haemoglobin is converted to biliverdin by the enzyme **heme oxygenase** (HOXG). Then NADPH comes into play and acts as the reducing agent, then molecular oxygen enters the reaction, out of this carbon monoxide (CO) is produced by oxidation of oxygen. Then iron is released from the molecule as a ferric ion (Fe^{3+}).

In addition, heme degradation is expected to be an evolutionary conserved response to oxidative stress. Briefly, when cells are exposed to free radicals, it induces rapid induction of the expression of the stress responsive heme oxygenase-1(Hmox1) isoenzyme which catabolizes heme. The reason why cells must increase exponentially is that their capability to degrade heme in response to oxidative stress remains unexplained but this appears to be the part of a cytoprotective response that supress the deleterious effects of free heme (Bosma and Chowdhury *et al*, 1995).

Bilirubin is transported into the liver by binding itself to a serum albumin and it gets conjugated with glucuronic acid which makes it more water soluble. The whole process is catalyzed by the enzyme **UDP-glucuronide transferase** (UDPGUTF). This form of bilirubin is excreted from the liver through bile in the form of bile salts. Then in the intestine the intestinal bacteria deconjugate bilirubin diglucuronide and converts bilirubin to urobilinogen. Some urobilinogen are absorbed by the intestinal cells and are transported into the kidneys and are

excreted out with urine which plays a major role in the yellow coloration of the urine. Then rest travels down the digestive tract and is converted to stercobilinogen in the large intestine. There it is oxidized to stercobilin, which responsible for the colour of faeces during its excretion through it.

2.3. HYPERBILIRUBINEMIA

It is a condition in which the normal blood bilirubin level becomes higher and that leads to deposition of bilirubin, as normally it is insoluble in blood; it is transported by conjugation with glucuronic acid which makes it soluble in the blood. **Hyperbillirubinemia** is a yellowing of the skin and other tissues of a new-born infant where if it crosses the required level of bilirubin range (table 1) than that will lead to symptomatic external appearance. A bilirubin level of more than 85 umol/l (5 mg/dL) manifests clinical jaundice in neonates whereas in adults a level of 34 umol/l (2 mg/dL) would look icteric (Weng and Chou *et al,* 2002).

Table 1: Clinical criteria of bilirubin range in different parts of the body to assess Neonatal Jaundice.

Area of the body	**Range of bilirubin (mg/100 ml)**
Face	4-8
Upper trunk	5-12
Lower trunk and thighs	8-16
Arms and lower legs	11-18
Palms and soles	>15

2.4. TYPES OF NEONATAL JAUNDICE

- **Physiological**:

When hyperbillirubinemia or Gilbert's syndrome happens due to any reason other than those concerning genetics or heredity and is caused due to a minor temporary defect during bilirubin

excretion or conjugation is called Physiological Jaundice. Irregularity in bilirubin metabolism at multiple step stages results in the occurrence of hyperbillirubinemia in the first few days of life of a neonate. The irregularities include:

- Increased bilirubin load on the hepatic cells for conjugation and defective uptake of it from plasma into liver cells.
- Defective conjugation of bilirubin.
- Decreased excretion by the body.
- Increased rate of entero-hepatic circulation in the liver.

❖ **Characteristics and symptoms of physiological jaundice**

- First it appears between 1-3 days of life.
- Maximum intensity observed on 7th day in preterm and 4-5th day in term neonates.
- Does not exceed 15 mg/ dl normally.
- Clinically not detectable after 14 days of life.
- Less treatment is essential but baby should be observed closely for signs of worsening jaundice.

Pathological jaundice:

When the disease is caused due any defect arising due to any deficiency of any vital enzyme due to a genetic defect that may not be reversible is called pathological jaundice.

Presence of any of the following signs indicates that it is pathological jaundice. Treatment is required in the form of exchange blood transfusion or phototherapy. Clinical jaundice detected before 24 hours of age shows the following characteristics.

- Rise in serum bilirubin levels by more than 5 mg/ dl/ day.
- Normal Serum bilirubin level more than 15 mg/ dl.
- Persistence of clinical jaundice beyond 14 days of life.
- Dark urine or clay/white colored stool and/or staining the clothes yellow.
- If direct bilirubin is more than 2 mg/ dl at any time (Maruo and Nishizawa *et al*, 2000).

2.5. CAUSES OF NEONATAL JAUNDICE

There are many different causes of jaundice, but since all of them are related to the liver they can be divided into three categories: Prehepatic, Hepatic, and Post hepatic in relation with the conjugation function of the liver, making bilirubin soluble by conjugation. These categories can broadly be called as causes of jaundice.

2.5.1. Prehepatic causes of jaundice

There are different prehepatic causes of jaundice. During hemolysis, haemoglobin is released into the bloodstream. When the rate of formation of new RBCs (erythropoiesis) and the rate of loss of old RBCs (hemolysis) are well balanced, the normally functioning liver can keep pace with disposal of haemoglobin produced during hemolysis. If the body has any difficulty in making RBCs (may be due to mineral or vitamin deficiencies), haemoglobin may leak into the circulation and overwhelm the liver. Conversely, if RBCs are destroyed at a rate higher than the normal one, the liver may also be overstressed. Disorders those are responsible for RBCs to dis-integrate prematurely are called hemolytic disorders.

A number of hereditary alterations hamper the red blood cells, including glucose-6-phosphate dehydrogenase (G6PD) deficiency (in which RBCs disintegrate when under stresses, particularly when exposed to certain drugs), sickle-cell anemia disease (in which the structure of haemoglobin is abnormal), and spherocytosis (in which presence of a protein in the outer membrane of the RBC causes weakness in the membrane) .

An enlarged spleen can also cause hemolysis. The spleen is the reservoir organ and the maintenance organ for the RBCs, which filters the blood. Its function is to filter out and destroy only worn-out RBCs of the body. If the spleen becomes enlarged then it also filters out normal cells as well that hampers normal metabolism. Haemolysis alone will rarely cause the total bilirubin level to rise above 7 mg/dL (Cappellini and Fiorelli, 2008). A wide range of conditions, mostly including the causes of haemolysis can enlarge the spleen to the point where it removes too many red blood cells from the body. In almost all causes of prehepatic jaundice, the predominant bilirubin is insoluble i.e. unconjugated.

2.5.2. Hepatic causes of jaundice

Liver diseases of all kinds, whether temporary or permanent, threaten the organ's ability to maintain bilirubin metabolism. Certain hereditary defects also affect the liver during bilirubin metabolism (Gilbert's syndrome and Crigler-Najjar syndrome), elevating the levels of unconjugated bilirubin in the blood. Also, there are several inherited conditions in which the liver cannot excrete bilirubin after it is made soluble (such as Dubin-Johnson syndrome and Rotor syndrome), resulting in direct (conjugated) bilirubin being the predominant form of the molecule. Unlike haemolytic causes of jaundice, the hepatic sources of jaundice often give mixed results (Luzzatto and Metha *et al,* 2001).

2.5.3. Posthepatic causes of jaundice

Posthepatic forms of jaundice include those caused when soluble bilirubin does not reach the intestines after leaving the liver, resulting in elevation of direct bilirubin levels. Such disorders are known as obstructive jaundices as they are obstructed in reaching their destination. The most common cause of obstructive jaundice is the presence of gallstones in the ducts of the biliary system of the human body. Some other causes include diseases where the bile ducts have been damaged, such as the autoimmune diseases like primary biliary sclerosis, lesions (benign or malignant) and trauma. Certain drugs (like anabolic and contraceptive steroids), and occasionally pressures caused due to a normal pregnancy,can cause the bile in the ducts to stop flowing. This process is called cholestasis (Tsai and Hung *et al,* 1998)

2.6. Clinical examination of jaundice

Originally described by Kramer, dermal staining of bilirubin can be used as a clinical guide to the levels of jaundice. Dermal staining in neonates progresses in the cephalo-caudal direction. The neonate needs to be examined in good daylight. The skin should be blanched with digital pressure and the underlying colour of skin and subcutaneous tissue should be noted.

New-borns detected to have yellow discoloration of the skin beyond the thighs should have an urgent laboratory confirmation for levels of bilirubin. Clinical assessment is unreliable if a new born has been receiving phototherapy and if the baby has dark skin.

2.6.1. Measurement of bilirubin levels

- **Biochemical:** The Laboratory estimation of total (unconjugated) and conjugated bilirubin based on Vanden Bergh reaction is the of the highest standard for bilirubin estimation.
- **Bilimeter:** This method is based on spectro-photometry ways and estimates total serum bilirubin of the blood. This method is very useful in neonates, as bilirubin is predominantly unconjugated.
- **Transcutaneous bilirubinometer:** This method is non-invasive and based on the principle of multi-wavelength spectral reflectance from the bilirubin staining in the skin. Variations due to skin thickness and pigmentation may interfere with the accuracy of the instrument.

2.7. GENETIC ASPECTS

2.7.1. UGT1A1 GENE

UDP-glucuronyltransferase also known as **UGT-1A** is an enzyme that in humans is encoded and controlled by the *UGT1A1* gene.UGT-1A is generally called as uridine diphosphate glucuronyltransferase (UDP-glucuronyltransferase, UDPGT) enzyme, it is an enzyme of the glucuronidation pathway in the liver that transforms small lipophilic molecules like hormones, steroids, bilirubin and drugs, into water-soluble and extractable metabolites.

The UGT1A1 (fig. 1) gene is the part of a complex locus that encodes several UDP-glucuronosyltransferases. The gene locus includes thirteen unique alternate first exons followed by four common exons. Out of all, four of the alternate first exons are considered as pseudo genes due to their suppressed nature. Each of the remaining nine 5' exons may be spliced into the four common exons which results in nine proteins with different N-termini and identical C-termini. Each first exon out of all encodes the substrate binding site, and is regulated by its own promoter (Watson and Gollan et al, 1989).

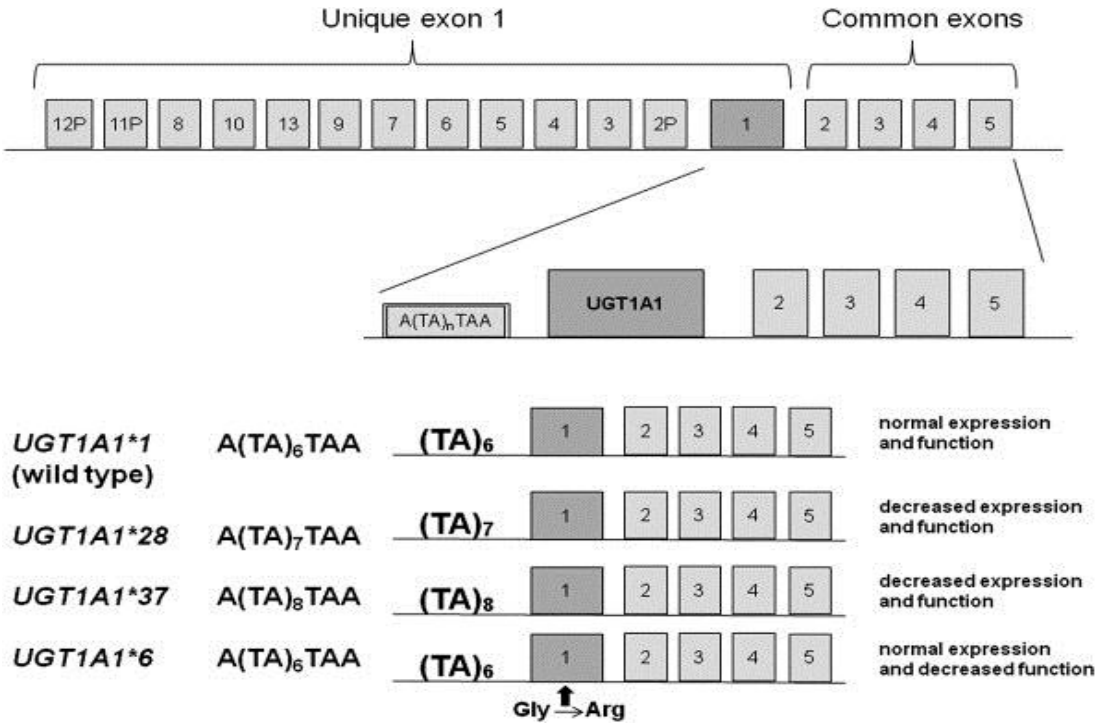

Fig 1: The promoter region of the UGT1A1 gene and its different types (Adapted from Watchko et al, 2009).

Mutations in this gene cause serious problems for bilirubin metabolism; each syndrome can be caused by one or many mutations and other genetic changes, so they are differentiated mostly by symptoms and not by particular mutations:

- **Gilbert syndrome** (GS) in many populations, other than those in southeast and east Asians and Pacific Islands, is commonly associated with a homozygous 2-bp insertion (TA) type mutation of the TATA box promoter region of the UGT1A1 gene, a polymorphism found in 40% of the alleles of many populations. About 10-15% of these populations are homozygous, but fewer (about 5%) actually develop UGT1A1-associated hyperbillirubinemia, so it indicates that this mutation alone may be a necessary but not sufficient factor in causing Gilbert's Syndrome, perhaps acting in combination with other UGT1A1 mutation(s) to increase the chances of getting GS. In all the populations mentioned above, as the TATA box mutation is much less common (about 3% of alleles), Gilbert's syndrome is more often due to missense mutations of the actual encoding region of the UGT1A1 gene. A specially designed phenobarbital-responsive enhancer module NR3 region (gtPBREM NR3) helps to increase UDPGT enzyme production, which

would make it conceptually and practically possible to medically control the blood bilirubin level (usually the level of total serum bilirubin in Gilbert syndrome patients vary from 1 to 10 mg/dL) (Bosma and Chowdhury *et al*, 1995.)

2.7.2. UDP-Glucuronosyl transferase Enzyme:

UDP-glucuronosyl transferase 1A1 (UGT1A1) is the key enzyme for bilirubin conjugation with glucuronic acid to make dissolvable in blood for its transport. Defects in this enzyme can cause a non-haemolytic unconjugated hyperbillirubinemia such as Crigler-Najjar syndrome classified as type 1 (CN 1) and type 2 (CN 2) along with Gilbert's syndrome. Since UGT1A1 is a very labile gene it is not measured by classical biochemical methods rather it is most suitably studied at the genetic level for non-hemolytic unconjugated hyperbillirubinemia. The cDNA of human *UGT1A1* gene was found to be located at chromosome 2q37 and was cloned in 1991. This led to the detection of genetic defects in patients with CN 1, CN 2, and Gilbert's syndrome. CN 1 and Gilbert's syndrome have been shown to be mainly associated with mutations in exons 2 to 5 and the promoter region, respectively (Babaoglu and Yigit *et al*, 2006).

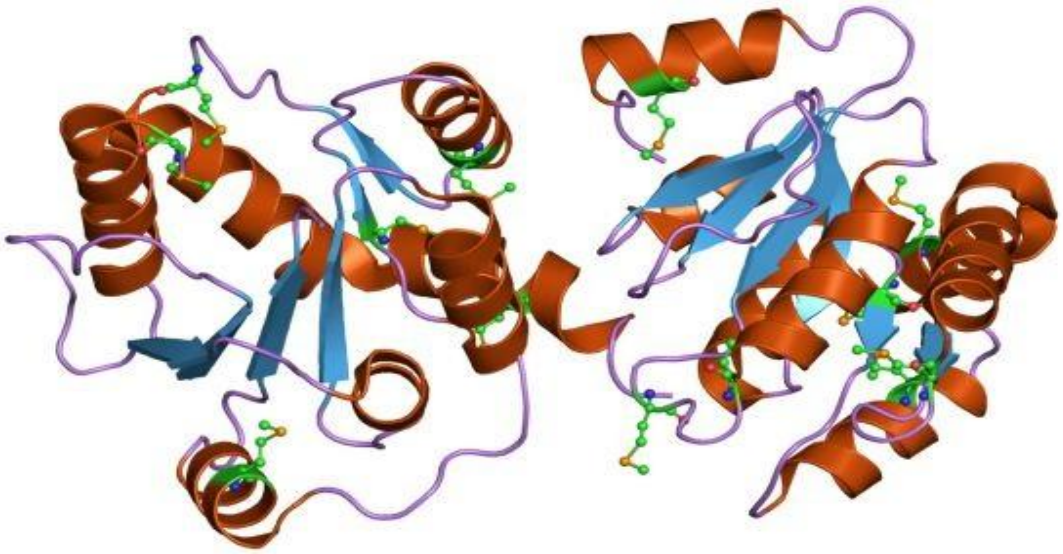

Fig 2: The 3D structure of the UDP glucuronosyl transferase enzyme (Adapted from Hoffmann et al, 1996)

The natural function of UGT1A1 is the catalysis of bilirubin glucuronidation. A genetic polymorphism in the UGT1A1 promoter (UGT1A1*28) results in the underexpression of the enzyme, causing an impaired bilirubin metabolism in the liver (i.e. reduced glucuronidation), clinically recognized as Gilbert's syndrome (UGT1A1 7/7 genotype). Sometimes due to a mutation in the UGT1A1 gene the UDP-glucuronosyl transferase enzyme is not produced which may result either due to an altered amino acid sequence or due a malformed domain of the enzyme protein which results in malfunctioning of the enzyme in conjugation of the bilirubin with glucuronic acid.

2.8. G6P DEHYDROGENASE DEFICIENCY

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that catalyses the first reaction in the pentose phosphate pathway, which provides reducing power to all cells in the form of NADPH. NADPH enables cells to counterbalance oxidative stress that can be triggered by many oxidant agent molecules, and preservation of the reduced form of glutathione. Since red blood cells do not have mitochondria, their only source of NADPH is the the pentose phosphate pathway; therefore, defence actions against oxidative damage is dependent on G6PD ability to carry out its function well. So the G6PD deficiency is a X-linked hereditary defect caused by mutations in the *G6PD* gene, which results in protein variants with different levels of enzyme activity, those are associated with a wide range of clinical and biochemical phenotypes (Ruwende and Hill *et al,* 1998).

The most common clinical manifestations are **neonatal jaundice or Gilbert's syndrome** and acute haemolytic anaemia, which in most cases are triggered in the body by an exogenous agent. The striking similarity between the areas where G6PD deficiency is common and *Plasmodium falciparum* malaria is endemic provides circumstantial evidence that G6PD deficiency confers resistance against malaria. The highest frequencies are seen in **Asia**, Africa, the Mediterranean population, and in the Middle East populations; owing to recent migrations. However, the disorder is also seen in North and South America and in northern European countries but is not common like the above regions.

2.8.1. Structure and function of G6PD

G6PD catalyses the first reaction in the pentose phosphate pathway, which converts glucose into the pentose sugars required for glycolysis and for various other biosynthetic reactions and processes. The pentose phosphate pathway also provides reducing power in the form of NADPH by the action of G6PD and 6-phosphogluconate dehydrogenase. NADPH also serves as an electron donor for many enzymatic reactions essential in biosynthetic pathways, and its production is essential for the protection of cells from oxidative stress. The enzyme G6PD is also necessary to regenerate the reduced form of glutathione that is produced with formation of one molecule of NADPH. As per some authors the reduced form of glutathione is essential for the reduction of hydrogen peroxide and oxygen radicals and the maintenance of haemoglobin and other red-blood-cell proteins in the reduced state.

The monomer of G6PD consists of 515 amino acids, with a molecular weight of around 59 kDa. The enzyme is active as a tetramer or a dimer, in a pH-dependent equilibrium condition. Every monomer consists of two domains: the N-terminal domain (amino acids 27–200), with a β–α–β dinucleotide binding site (amino acids 38–44); and a larger β+α domain, consisting of an antiparallel nine-stranded sheet like structure. The dimer interface lies in a barrel arrangement in the second part of the G6PD molecule (Cappellini and Fiorelli *et al*, 2008). The two domains are linked by a helix, containing the totally conserved eight-residue peptide that acts as the substrate binding site (amino acids 198–206). Viewing the structure, at 3 A (0·3 nm) resolution, reveals an NADP+ (a coenzyme) molecule in every subunit of the tetramer which is distant from the active site but very close to the dimer interface in the structure. Constant stability of the active quaternary structures is essential for normal G6PD activity.

3. MATERIALS AND METHODS

The materials and methods that were used are as follows:

3.1. TISSUE SAMPLING AND RNA EXTRACTION.

3.1.1. Tissue Sampling

Blood samples were collected from the Ispat General Hospital (IGH) Rourkela, Odisha, from neonatal jaundice patients and were then stored in ice and immediately processed for better RNA extraction.

3.1.1. RNA extraction

Total RNA was extracted from the neonatal jaundice patients' blood samples using GeneJET^{TM} RNA Purification Kit (Fermentas).

- **Extraction from Blood:**

The collected blood was centrifuged at 3000 rpm for 15 mins at 4° C. The supernatant containing the serum was separated from the pellet which contains the blood cells. The pellet was resuspended in 600 µl of Lysis Buffer (supplemented with 20 µl of 14.3 M β-mercaptoethanol for every 1ml of Lysis Buffer) and was vortexed for through mixing. 450 µl of ethanol (96-100%) was then mixed with the working solution. Around 700 µl of the lysate was transferred to a GeneJET^{TM} RNA Purification Column inserted in a collection tube and was centrifuged at 12000 rpm for 1 min at 4° C. The flow-through was discarded and the column was placed into a new 2 ml RNase-free microcentrifuge sterilized tube. Then 700 µl of Wash Buffer 1(supplemented with 250 µl of ethanol for every 1ml Wash buffer 1) was added to the tube column and was then centrifuged for 1 min at 12000 rpm. The flow-through was discarded and 600 µl of Wash Buffer 2 (supplemented with 850 µl of ethanol for every 0.5 µl Wash buffer 2) was added to the column. It was centrifuged at 12000 rpm for 1 min at 4° C. The flow-through was again discarded as a waste. Then centrifugation was again again done at 12000 rpm for 1 min at 4 ° C after adding 250 µl of Wash buffer 2 in the solution. The flow-through was discarded and the column was transferred to a sterile 1.5 RNase-free sterilized microcentrifuge

tube. 100 µl of nuclease-free water was then added to the column and centrifuged for 1 min at 12000 rpm to elute RNA. The RNA was stored at - 20° C for further use or was immediately processed for cDNA synthesis.

3.2. RNA QUANTIFICATION BY UV SPECTROPHOTOMETRY

The concentration of the extracted total RNA from the blood was quantified by measuring the absorbance at 260 nm in a spectrophotometer (ELICO, BL 200 Bio Spectrophotometer, double beam) and calculated by using the formula as given below:

$$\text{Total RNA (µg /ml)} = OD_{260} \times 40 \times \text{Dilution factor.}$$

3.2.1. Quantitative Estimation of RNA Concentration by Denaturing Gel Electrophoresis

The extracted RNA from the blood sample was run on a denaturing agarose gel and the quantity of RNA estimated from the band intensity of the RNA. For denaturation gel (40 ml), 0.6 g agarose (Sigma), 28.8 ml dH_2O (Sigma), 7.2 ml formaldehyde (Sigma), 4 ml 10X MOPS buffer were mixed properly. About 2 µl (2µg) of the total RNA was mixed with 18 µl 1X Reaction Buffer (2µl of 10X MOPS Buffer,10 µl formamide (Sigma) ,2 µl 0.2 mg/ml Etbr (Sigma), 4 µl formaldehyde) and incubated at 55 °C for 1 hr. It was then cooled on ice and loaded in the wells of the denaturing gel for electrophoresis.

3.2.2. cDNA synthesis

Total RNA (4 µg) from blood sample were used for first strand cDNA synthesis by reverse transcription using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas) in a thermo cycler (Biorad). The RNA was incubated with 1 µl of oligo (dT) primers (100 µM, 0.2 µg/µl) and 12 µl of nuclease-free water at 65 °C for 5 min. The reaction was then cooled on ice cubes to allow the primers to anneal to the RNA and was then spun down and placed on ice again after which the following comp

onents were added to the reaction in order; 4 µl of 5X Reaction Buffer, 1 µl of Ribolock^{TM} RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs and 1.0 µL of RevertAid^{TM} M-MuLV-Reverse Transcriptase (200 U/µl). The reagents were gently mixed and incubated at 42°C for 1 hr. Then heating the solution at 70°C for 5 min terminated the reaction and the synthesized cDNA was stored at –20 ^{0} C for further use.

3.3. PCR AMPLIFICATION OF UGT1A1 GENE:

The polymerase chain reaction (PCR)–restriction method was used to amplify the gene and study the variant sites in the UGT1A1 gene in the patients

The primers used:

Sense: 5′ GTCACGTGACACAGTCAAAC 3′
Antisense: 5′GCTTGCTCAGCATATATCTGG 3’.

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3.3.1. The Method:

The PCR mixture consists of 20ml of the following Constituents:

- 200 ng of DNA
- 20 ng of each primer
- 1.25 mM of each dNTP (3.2 mL)
- buffer solution (100 mM of Tris-HCl)
- 500 mM of KCl, 15 mM of MgCl2
- 0.01% gelatin
- 0.4 U of thermostable DNA polymerase (taq polymerase).

Autoclaved PCR tubes were labelled according to the samples for the study. A master mix of 1 reaction was prepared in a sterilize eppendroff tube by adding 40.8µl of autoclaved Millipore

water, 5µl of 10xTaq assay buffer, 1 µl forward primer,1 µl reverse primer, 1 µl dNTPs and 0.2 µl of TaqDNA polymerase, then mixed properly by short spin and kept it in ice.1 µl cDNA was taken from each sample and was put into the labelled PCR tube accordingly, Keep one negative control (without cDNA).The tubes were tapped gently and spun for a few seconds. After this the tubes were placed in thermal cycler with program set as follows for UGT1A1 gene:-

94c for 1 min (Initial denaturation)	
94c for 20 sec (Denaturation)	
55c for 1 min (Annealing)	35 cycles
72c for 1 min (Extension)	
72c for 10 min (Final extension)	
40c hold on forever	

The PCR amplification was performed in a DNA thermal cycler for 35 cycles of denaturation for 1 minute at 94C, annealing for 1 minute at 55C, primer extension for 1 minute at 72C, and a final extension for 10 minutes at 72C.

The PCR product was digested with the appropriate restriction enzyme and analyzed on a 3% agarose gel. Then the gel was documented through a gel documentation system and then the photo document was analysed for the results.

4. RESULTS AND DISCUSSIONS

4.1. RESULTS:

What we found from the experiment was that unfortunately we never found any mutated gene or a non-expressing gene from any of the 3 samples of the patient neonates. This was due to the less number of samples taken for the study.

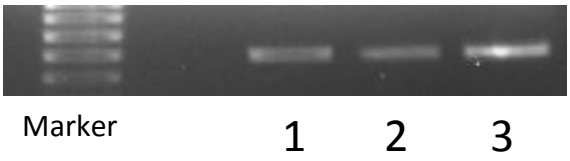
Table 2: The expression profile along with other birth information of the three patients.

SL. NO.	SAMPLE	SEX OF THE NEONATE (Newborn)	Wt. AT BIRTH (gms. Apprx.)	AGE (in days)	STATUS OF DELIVERY	UGT1A1 EXPRESSION PROFILE
1.	A	MALE	1300	15	NORMAL	EXPRESSED
2.	B	MALE	1250	21	NORMAL	EXPRESSED
3.	C	FEMALE	1400	10	CAESAREAN	EXPRESSED

From the above result table (Table 2) we could see that out of the 3 neonates 2 were males and one female and 1 baby was delivered through caesarean surgery

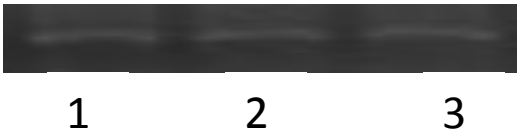
But all these factors had no impact on the expression of the UGT1A1 gene as in each case it was expressed properly even though the babies had hyperbillirubinemia (kernicterus). In some cases in a premature baby there happens to be signs showing neonatal jaundice or hyperbillirubinemia but then that is always due to metabolic disorders making it physiological type jaundice and never a pathological as the expression of the UGT1A1 gene has little to do with the condition.

THE GEL-DOC PHOTOS



β ACTIN IN NEONATAL JAUNDICE

Fig 3: The Gel doc photo of β actin expression in the 3 experimental samples.



UGT1A1 GENE

Fig 4: Gel doc photo of UGT1A1 gene expression in the 3 experimental samples.

From above photos showing the results of gel running of the cDNA clearly indicates the expression profile of the β actin of the housekeeping gene in the blood samples of the neonatal jaundice patients (Fig 1) and the expression profile of the UGT1A1 gene of the same samples (Fig 3) to see that both the genes are expressed normally in the patients.

4.2. DISCUSSION

The reason behind the proper expression of the UGT1A1 gene even though the neonates had jaundice is that the neonates were suffering from physiological jaundice which has no relation with the UGT1A1 gene for the disease to occur and is more of an adaptive measure taken by the body to acclimatize with the sudden change in the environment with increased oxygen supply.

In physiological jaundice, as there is excessive hemolysis occurring in the liver, which is a beneficiary process to get rid of the extra nonfunctional RBCs, so much bilirubin is produced that the liver finds it difficult to excrete through conjugation as a result there is presence of excess bilirubin in the blood which being less soluble gets deposited underneath the skin. The reason behind the condition is more due to physiological disorders and not due to underexpression of the UGT1A1 gene which is responsible for the proper function of the UDP glucuronosyl transferase enzyme which plays a major role in bilirubin conjugation as is evidenced by the expression profile of the gene as shown by the above gel doc photos (Fig 3) which clearly shows a expressed gene seen in the gel.

5. CONCLUSION

Neonatal Jaundice is a rarely pathological and often physiological disease. The pathological reasons are due to some genetic or epigenetic defects of the gene. This disease provides vital reasons for exploration as it is concerned with a neonate. This study helped in revealing some truth by bringing out the expression profile of the UGT1A1 gene which was done through RNA isolation, cDNA synthesis and PCR amplification studies of the gene but the number of samples taken is very less to confirm the inference to hold ground which provides enough scope for researchers to explore a lot more in this field of study. In all the 3 samples the gene was expressed suggesting for an assay taking a large no of samples, as the gene is rarely defective.

This field provides enough scope and curiosity for the researchers to work on the mutations that may happen, which may lead to find some ways to heal the defect render the patient disease less forever.

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